(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 October 2004 (14.10.2004)

PCT

(10) International Publication Number
WO 2004/087891 A1

(51) International Patent Classification[7]: C12N 1/20, A61K 35/74, A23C 9/152, A23L 1/30, A23C 9/123, C12R 1/225

(21) International Application Number: PCT/IB2003/001739

(22) International Filing Date: 2 April 2003 (02.04.2003)

(25) Filing Language: English

(26) Publication Language: English

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: LACTOBACILLUS FERMENTUM STRAIN AND USES THEREOF

(57) Abstract: The present invention concerns a new strain of *Lactobacillus fermentum* (LB-f) that is useful in preventing or treating gastrointestinal disorders in mammals, especially in humans. Also, this strain can be used as a dietary product that is beneficial to the wellbeing and health of mammals, including humans.

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LACTOBACILLUS FERMENTUM STRAIN AND USES THEREOF

The present invention relates to a new strain of the *Lactobacillus* genus, and uses thereof for medical or dietary purposes.

In particular, the invention concerns a new strain of *Lactobacillus fermentum* (LB-f) that is useful in preventing or treating gastrointestinal disorders in mammals, especially in humans.

Also, this strain can be used as a dietary product that is beneficial to the wellbeing and health of mammals, including humans.

Microorganisms, and more particularly bacteria, that produce lactic acid as a major metabolic compound have been known for a long time. These bacteria may be found in milk, in milk processing factories, in living or decaying plants, as well as in the intestine of mammals, especially humans. These microorganisms, brought together under the generic formula « lactic acid bacteria », represent a rather inhomogeneous group and comprise e.g. the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Pediococcus*, etc ...

Lactic acid bacteria have been utilized as fermenting agents for the preservation of food, taking benefit of a low pH and the action of fermentation products generated during the fermentative activity thereof to inhibit the growth of spoilage bacteria. In this context, lactic acid bacteria have been used for preparing a variety of different foodstuff such as cheeses, yogurts and other fermented dairy products from milk.

Quite recently, lactic acid bacteria have attracted a great deal of attention in that some strains have been found to exhibit valuable properties to mammals, including humans, upon ingestion. In particular, specific strains of the genus *Lactobacillus* or *Bifidobacterium* have been found to be able to colonize the intestinal mucosa and to assist in the maintenance of the wellbeing of mammals.

For instance, European patent application EP 0 203 586, in the name of Pioneer Hi-Bred International, discloses a composition for treating gastrointestinal diseases, e.g., caused by enterotoxigenic strains of *Escherichia coli*, in animals, said composition containing *L. fermentum* ATCC 53113 or mutants thereof. Animals as referred to therein are domestic animals, such as pigs, cows, sheep, goats, and horses. *L. fermentum* ATCC 53113 was isolated from the gut of a healthy newborn pig.

During the last few years, research has also focused on the potential use of lactic acid bacteria as probiotic agents.

Probiotics are considered to be viable microbial preparations which promote the health of mammals, especially humans. Probiotics are deemed to attach to the intestine's mucosa, colonize the intestinal tract and likewise prevent attachment of harmful microorganisms thereon. A crucial prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

In particular, European patent application EP 1 034 787, in the name of Société des Produits Nestlé S.A., discloses new strains belonging to the *Lactobacillus* genus, that are useful for preventing diarrhoea, by inhibiting intestine colonization by pathogenic bacteria. These strains, especially *L. paracasei* CNCM I-2116, can be used for preparing pharmaceutical or dietary compositions.

Furthermore, International patent application WO 02/45727, in the name of Plbio Co., Ltd., describes lactic acid bacteria capable of inhibiting activities and growth of *Helicobacter pylori* causing stomach ulcer and adhesion to the gastric mucosa. The lactic acid bacteria disclosed therein as being able to suppress stomach ulcers, are selected from the group of *L. coprophilus*, *Enterococcus durans*, *Streptococcus faecalis*, and

L. fermentum. According to this disclosure, the lactic acid bacteria can be used in pharmaceutical compositions, in cosmetic preparations, for instance for treating acne, as well as in food additives, that can be added to, e.g., yogurts, dairy goods, cheeses, and the like.

In view of their potential properties, strains of lactic acid bacteria appear to be very valuable both for medical and dietary purposes.

In this context, a strain belonging to the *Lactobacillus* genus, namely *L. acidophilus* LB, and exhibiting interesting anti-diarrhoea properties, is currently used in France in the commercially-available "Lactéol®" pharmaceutical compositions. Nevertheless, this LB strain has a number of drawbacks related to the very specific conditions required to culture. Among these conditions, the composition of the culture medium should be mentioned, this medium having to be enriched by compounds from bovine origin such as lactoserum, bovine peptone, and casein. Moreover, before cultivating the LB strain, it is required to implement preliminary steps for appropriately preparing the culture medium by sterilization and filtration thereof, so that a clarified culture medium is obtained. Also, cultivating the LB strain is a relatively long and costly process, with high energetic demands; it produces a lot of organic waste that has to be collected and treated.

Therefore, there is a desire in the art for providing new strains of lactic acid bacteria that are at least as beneficial to the wellbeing and health of mammals, especially humans, as is the LB strain, but that have advantageous features compared thereto, especially in terms of conditions of culture.

To this end, the present invention provides a novel microorganism, namely a lactic acid bacterial strain belonging to the genus *Lactobacillus*, having the capability of preventing colonization of the stomach and the intestine by pathogenic bacteria, responsible for gastrointestinal disorders, and being easy to cultivate for industrial purposes.

The *Lactobacillus* strain of the invention is thus valuable for the wellbeing and health of mammals, including animals and humans, preferably humans, and more preferably infants.

In this respect, said *Lactobacillus* strain is a biotherapeutic agent, i.e., a biological agent which exhibits a therapeutic activity of interest.

Moreover, the *Lactobacillus* strain of the present invention is of high interest as far as safety standards are concerned, especially with regard to potential contaminating viral agents or unconventional transmissible pathogens. In this connection, said strain can be consumed or administered, as described hereunder, by mammals, more particularly by humans, without any risk.

In a first aspect, the present invention relates to a *Lactobacillus fermentum* strain (LB-f strain), deposited at the CNCM (Paris, France) on March 27, 2003, under registration number I-2998.

This LB-f strain is herein described by phenotypic and genotypic features.

On the one hand, the LB-f strain exhibits at least the following phenotypic characters:

- regular, non sporing, Gram-positive rod;
- heterofermenting;
- catalase negative;
- L (+)-lactic acid-producing.

On the other hand, the LB-f strain is genotypically characterized by a sequence of 16S ribosomal DNA (rDNA), which comprises a nucleotide sequence selected from:

- SEQ ID No. 1 ;
- its complementary sequence ; and
- sequences identical at least at 98.1% to SEQ ID No. 1 or to its complementary sequence.

As used herein the term "complementary" means that, for example, each base of a first nucleotide sequence is paired with the complementary base of a second nucleotide sequence whose orientation is reversed. The complementary bases are A and T (or A and U) or C and G.

The terms « nucleic acids » and « nucleotide sequences » are used interchangeably according to their conventional meaning in the technical field of the invention.

According to a first embodiment, nucleotide sequences also encompassed by the present invention are identical at least at 98.5%, and preferably at least at 99% to SEQ ID No. 1 or to its complementary sequence.

According to a second embodiment, these nucleotide sequences are identical at least at 99.5%, and preferably at least at 99.8% to SEQ ID No. 1 or to its complementary sequence.

By "sequence identity", it is herein referred to the identity between two nucleic acids.

Sequence identity can be determined by comparing a position in each of the two nucleotide sequences which may be aligned for the purposes of comparison. When a position in the compared sequences is occupied by the same base, then the sequences are identical at that position. A degree of sequence identity between nucleic acid sequences is a function of the number of identical nucleotides at positions shared by these sequences. Since two nucleotide sequences may each (i) comprise a sequence (i.e., a portion of a complete nucleotide sequence) that is similar, and (ii) may further comprise a sequence that is divergent, sequence identity comparisons between two or more nucleotide sequences over a "comparison window" refers to the conceptual segment of at least 20 contiguous nucleotide positions wherein a nucleotide sequence may be compared to a reference nucleotide sequence of at least 20 contiguous nucleotides and wherein the portion of the nucleotide sequence in the comparison window may comprise additions or deletions

(i.e. , gaps) of 20 percent or less compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison. For example, gaps can be introduced in the sequence of a first nucleic acid sequence for optimal alignment with the second nucleic acid sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, the nucleic acids are identical at that position.

The percent identity between the two sequences is a function of the number of identical positions shared by the sequences.

Hence % identity = [number of identical positions / total number of overlapping positions] X 100. The percentage of sequence identity is thus calculated according to this formula, by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions (the "number of identical positions" in the formula above), dividing the number of matched positions by the total number of positions in the window of comparison (e.g., the window size) (the "total number of overlapping positions" in the formula above), and multiplying the result by 100 to yield the percentage of sequence identity.

In this comparison, the sequences can be the same length or may be different in length. Optimal alignment of sequences for determining a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsh (1972), by the search for similarity via the method of Pearson and Lipman (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin

Genetics Software Package Release 7.0, Genetic Computer Group, 575, Science Drive, Madison, Wisconsin), or by inspection.

The best alignment (i.e., resulting in the highest percentage of identity over the comparison window) generated by the various methods is selected.

The percentage of sequence identity of a nucleic acid sequence to a nucleotide sequence of reference can also be calculated using BLAST software (Version 2.06 of September 1998) with the default or user defined parameter.

In a second aspect, the present invention concerns a method for cultivating a LB-f strain as defined above, comprising at least:

a) providing a culture medium containing at least lactose and yeast extract;

b) cultivating said LB-f strain in said culture medium under fermenting conditions; and

c) recovering the thus obtained culture of the LB-f strain.

All the steps of such a method are conventional in the technical field of the invention, and can be performed by a skilled artisan in the light of his general knowledge.

Interestingly, a suitable culture medium is free of compounds from bovine origin. Such a medium free of derivatives from bovine origin ensures enhanced safety of the thus obtained culture of LB-f strain.

Advantageously, the method for cultivating the LB-f strain is simple, easy to perform, and does not require sterilization and filtration of the medium prior to culture. Moreover, waste products are considerably reduced.

According to a first embodiment, said culture medium contains lactose at a concentration range of about 50 to about 100 g/l.

According to a second embodiment, said culture medium contains yeast extract at a concentration range of about 5 to about 20 g/l.

According to a third embodiment, said fermenting conditions in step b) are pH-regulated, said pH ranging between about 4.5 and 5.5.

Also encompassed by the second aspect of the invention is a method as described above, further comprising separating the biomass from the culture supernatant (LB-f-SCS) by centrifugating said culture of LB-f strain recovered in step c).

Yet encompassed by the second aspect of the invention is a method as described above, wherein, once centrifugation has been performed, said biomass and/or said LB-f-SCS are recovered.

In a third aspect, the present invention is directed to a *Lactobacillus fermentum* culture supernatant (LB-f-SCS) obtainable by the aforementioned method for cultivating a LB-f strain.

In a fourth aspect, the invention is related to a LB-f strain or a LB-f-SCS as defined above, for use as a medicine.

In particular, said medicine is used for preventing and/or treating gastrointestinal disorders.

Of great significance is the fact that the medicine according to the invention does not necessitate the supplemental use of antibiotics and relies rather upon "natural" mechanisms of controlling the pathogens, in particular by preventing same from binding to gut-associated tissue.

This is significant in the face of growing public concern over the misuse of antibiotics, and the effect that over-intake of antibiotics is given to have on the population's health.

The gastrointestinal disorders against which the present invention is effective can be any in which the underlying etiology is microbial, for example, bacterial or viral in nature.

Alternatively, the present invention is also useful in mammals, including humans, where the normal gut flora has been eliminated or unbalanced, for example, following severe viral gastroenteritis or high dose antibiotic therapy, in order to aid in the restoration of the normal gut flora and prevent colonization by opportunistic pathogens.

Preferably, in the context of the invention, the expression "gastrointestinal disorders" refers to disorders or diseases that are selected from ulcers and infections due to *Helicobacter pylori*, intestinal inflammatory diseases, such as ulcerous colitis, Crohn's disease and pouchitis, irritable bowel syndrome, steatohepatitis, hepatic steatosis, and infectious diarrhoea.

In a fifth aspect, the invention concerns the use of a LB-f strain or a LB-f-SCS as described above, for the manufacture of a medicine for preventing and/or treating gastrointestinal disorders.

In a sixth aspect, the present invention relates to the use of a LB-f strain or a LB-f-SCS as disclosed herein, as a dietary product.

In a seventh aspect, the invention is directed to a pharmaceutical composition comprising a LB-f strain or a LB-f-SCS, and a pharmaceutically acceptable carrier.

The composition of the present invention is highly desirable in that the LB-f strain of the invention is non-pathogenic and should thereby render unlikely the occurrence of any deleterious effects due thereto.

According to a first embodiment, said LB-f strain is present in the pharmaceutical composition an amount from about 10^9 to about 10^{12} bacteria/g, preferably from about 10^9 to about 10^{11} bacteria/g, and more preferably from about 10^9 to about 10^{10} bacteria/g.

According to a second embodiment, said LB-f-SCS is present in the pharmaceutical composition in an amount of at least about 100 mg per gram of composition.

According to a third embodiment, the pharmaceutical composition of the invention is ingestible.

Such an ingestible composition is preferably in a form selected from tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding, wet tube feeding.

According to an eighth aspect, the present invention is related to a method for treating or preventing gastrointestinal disorders in mammals, especially humans, that are in need of such treatment.

This method comprises administering to a mammal in need of such treatment a pharmaceutically effective amount of a medicine selected from the group of:

- a LB-f strain as defined above; or
- a LB-f-SCS as described herein; or
- a pharmaceutical composition as previously mentioned.

As used herein, the expression "in need of such treatment" refers to a mammal, including a human, having, or being at risk of having, gastrointestinal disorders.

According to a first embodiment, administration of said medicine is performed orally.

To do so, a suitable medicine is in a form selected from tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding, wet tube feeding.

In a ninth aspect, the present invention concerns a dietary composition comprising a LB-f strain or a LB-f-SCS as defined above, and a food carrier, such as milk, cheese, yogurts, and the like.

According to a first embodiment, said LB-f strain is present in the dietary composition in an amount from about 10^5 to about 10^9 bacteria/g, preferably from about 10^6 to about 10^8 bacteria/g, and more preferably from about 10^6 to about 10^7 bacteria/g.

According to a second embodiment, said LB-f-SCS is present in the dietary composition in an amount of less than about 100 mg per gram of composition.

A dietary composition according to the present invention is advantageously ingestible.

In this respect, said ingestible composition is preferably selected from milk, yogurt, curd, cheese, fermented milks, fermented milk-based

products, ice-creams, fermented cereal-based product, milk-based powders, infant formulae.

Generally, compositions of the present invention, either pharmaceutical or dietary, may be in a liquid, solid, lyophilized, or gel form.

On the one hand, in solid oral dosage forms, the compositions may comprise the LB-f strain or the LB-f-SCS, together with an appropriate carrier, that is either a pharmaceutically acceptable carrier or a food carrier. Such a carrier may be in a form chosen among: aqueous or non-aqueous liquids, and solids.

Also, the solid compositions may contain inert diluents such as sucrose, lactose, starch, or vermiculite, as well as lubricating agents. Lubricating agents help the compositions to pass through the gut.

In the case of capsules, tablets and pills, the unit dosage forms may also comprise buffering agents.

Others forms of oral administration may also be prepared with a gastric or an enteric coating which would prevent dissolution of the compositions until reaching the stomach or the intestines, respectively.

On the other hand, liquid dosage forms for oral administration may comprise an enterically-coated capsule containing the liquid dosage form.

Suitable liquid forms include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water, sugars, polysaccharides, silicate gels, gelatin, or an alcohol. Inert diluents do not actively participate in the therapeutic or dietary effect of interest. Besides the inert diluents, such compositions can also include adjuvants, for instance wetting, emulsifying, suspending, sweetening, flavouring, and perfuming agents.

Those of ordinary skill in the art will know of other suitable diluents and dosage forms, or will be able to ascertain such, using routine experimentation.

Further, the administration or ingestion of the compositions of the invention can be carried out using standard techniques common to those of ordinary skill in the art.

The present invention is illustrated, without being limited, by the following drawings.

Figure 1: Graphic representation of *Lactobacillus fermentum* LB-f adhesion to Caco-2/TC7 cells.
A: living LB-f (1.10^9 bacteria/well)
B: living LB-f (2.10^8 bacteria/well)
C: killed LB-f (2.10^9 bacteria/well)
D: killed LB-f (1.10^9 bacteria/well)

Figure 2: Graphic representation of inhibition of cell-association of strain DAEC C1845, compared to controls of cell-association performed in:
A: DMEM
B: MRS-HCl pH 4.5
1: living LB
2: living LB-f
3: killed LB
4: killed LB-f

Figure 3: Graphic representation of invasion of *Salmonella* serovar Typhimurium in the presence of *Lactobacillus* (bacteria and spent culture).
A: SL1344 control
B: living LB-f
C: killed LB-f

Figure 4: Graphic representation of viability, adhesion and invasion of SL1344 after contact with culture supernatants during 1 hour.

A: DMEM control
B: MRS control
C: LB-SCS unheated
D: LB-f-SCS unheated
E: LB-SCS heated at 100°C
F: LB-f-SCS heated at 100°C

Figure 5: Graphic representation of inhibition of viability and adhesion of DAEC C1845, after contact during 1 hr with culture supernatants LB-f-SCS, compared to DMEM and MRS controls.
A: DMEM control
B: MRS control
C: LB-f-SCS unheated
D: LB-f-SCS heated at 100°C

Figure 6: Graphic representation of *H. pylori* viability after contact during 2 hrs with LB-f-SCS, compared to BHI and MRS controls.
A: BHI control
B: MRS control
C: LB-f-SCS unheated
D: LB-f-SCS heated at 100°C

Figure 7: Graphic representation of development of urease activity after contact during 2 hrs with *H. pylori*.
A: BHI control
B: MRS control
C: LB-f-SCS unheated
D: LB-f-SCS heated at 100°C

Figure 8: Graphic representation of cell-invasion of *Salmonella* SL1344 after treatment for 1 hr with LB-f-SCS, said treatment being performed after infection, compared to DMEM control.
A: DMEM control
B: LB-f-SCS unheated
C: LB-f-SCS heated ay 100°C

Figure 9: Graphic representation of cell-adhesion of DAEC C1845 after treatment for 1 hr with LB-f-SCS, said treatment being performed after infection, compared to DMEM and MRS controls.
A: DMEM control
B: MRS control
C: LB-f-SCS unheated
D: LB-f-SCS heated at 100°C

The above disclosure generally describes the present invention.

A more complete understanding can be obtained by reference to the following experimental procedures and results which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

I. EXPERIMENTAL PROCEDURES

I.1 Pathogens:

Salmonella typhimurium SL 1344 (Finlay and Falkow, 1997) was a gift of B.A.D. Stocker (Stanford, California), *Escherichia coli* C1845 was a gift of S. Bilge (University of Washington, Seattle); enterotoxigenic *E. coli* (ETEC) strains H10407 expressing Coli Factor Adhesin type 1 (CFA/I) was provided by A. Darfeuille-Michaud (Faculté Médecine-Pharmacie, Clermont-Ferrand).

E. coli strains were grown on CFA-agar containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract, 0.005% magnesium sulfate, and 0.0005% manganese chloride in 2% agar for 18 hours at 37°C.

For radiolabeling, bacteria were subcultured twice at 37°C for 24 hours in CFA or Trypticase Soya Agar (TSA) broth. They were metabolically labeled by the addition of ^{14}C-acetic acid (Amersham, 94 mCi/mmol; 100 µCi per 10ml tube).

S. typhimurium was cultured at 37°C for 18 hours in Luria broth and an exponential culture of *Salmonella* was used for assays.

For radiolabeling assays, *S. typhimurium* was cultured 4 hours in Luria broth and then was subcultured at 37°C for 45 min in methionine medium (Difco) with ^{35}S-methionine (Amersham 1000 Ci/mmol, 20 µCi/ ml) for radiolabelling.

Strain of *H. pylori* was provided by I. Corthesy-Theulaz (Institute of Microbiology, Lausanne University, Lausanne, Switzerland). *H. pylori* strain 1101 was isolated from a patient suffering from functional dyspepsia and erosive gastritis (Corthézy-Theulaz et al., 1995).

H. pylori was grown on Brain-Heart Infusion (BHI)-agar plates containing 0.25% yeast extract (Difco Laboratories), 10% horse serum. *Helicobacter* culture was incubated upside down in a gas jar with microaerophilic atmosphere (Gas-generating kit, CampyGen, Oxoid Ltd) at 37°C for 36 hours.

I.2 Cultured cell lines:

In the last few years, human intestinal cell lines have been established. These include HT-29 and Caco-2 cells established in 1964 and 1974, respectively, by Jorgen Fogh at the Memorial Sloan Kettering Cancer

Center (New York, USA) (Fogh et al., 1977), which are both derived from human colonic adenocarcinomas. The clone Caco-2/TC7 was used here (Chantret et al., 1993). They display spontaneously in culture (Caco-2 and Caco-2/TC7) the following types of enterocyte differentiation specific to the human small intestine (Zweibaum et al., 1991):

- cell polarisation
- development of an apical brush border and tight junctions
- expression of intestinal hydrolases, e.g.: saccharase-isomaltase (SI), neutral aminopeptidase (NAp), dipeptidyl peptidase IV (DPP IV)...,
- production of structural proteins: villin...,
- presence of basolateral receptors: Vaso Active Intestinal Peptide (VIP), alpha2-adrenergic receptor...,
- presence of transport proteins: glucose transporter and transepithelial hydroelectrolytic transport.

Cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (DMEM) (25 mM glucose) (InVitrogen, Cergy-Pontoise, France), supplemented with 15% heat-inactivated (30 min, 56°C) fetal calf serum (InVitrogen, Cergy-Pontoise, France) and 1% non-essential amino acids (InVitrogen, Cergy-Pontoise, France). Cells were used at post-confluence after 15 days of culture (differentiated cells) for cell-association and cell-invasion assays using *S. enterica* serovar Typhimurium.

I.3 Lactobacilli adhesion:

The required concentration of the bacterial suspension was made once they had been counted on a Petit Salumbéni cell. The cell layers were washed twice (2 ml) with sterile PBS. One millilitre of DMEM was placed on the cell layers and the same volume of the bacterial suspension was then added. They were left in contact for one hour with 10 % CO_2 to preserve the integrity of the cell layer. The medium was removed at the

end of incubation and the cell layers were washed five times (2 ml) with phosphate-buffered saline (PBS).

In each wash the well dishes were shaken round twenty times to remove non-adherent bacteria. Previous experiments have shown that further washes do not significantly reduce the adhesion ratio. The cell layers were then fixed in the following series of methanol baths: 70°C 10 min, 95°C 10 min and 100°C 15 min. The cell layer were then dried, stained (Gram), dehydrated in xylene and mounted for microscopic observation (Eukitt). Each test was duplicated for three successive passages. The adherent bacteria were counted in 20 randomly chosen microscopic fields. The adherent bacteria were counted by two different technicians to prevent errors. Adhesion of *Lactobacillus* was graded on a 0 to 4 scale: 0, absence; ++, reduced number; +++, large number; ++++, high number.

LB-f strain was killed by incubating at 110°C for 1 hour (see Figure 1).

I.4 Association and intracellular inhibition of enterovirulent bacteria with Lactobacilli and spent culture supernatant:

- Inhibition association was conducted as follows :

The Caco-2/TC7 monolayers were washed twice with PBS. ^{14}C-radiolabelled bacteria were suspended in the culture medium.

For *E. coli* assays, incubations were conducted in presence of 1% D-mannose that inhibits type 1 pili adhesion. For evaluation of adhesion interference 250 µl of radiolabelled bacteria (4.10^8 CFU cells/ml), 250 µl of living or heat-killed *L. fermentum* LB-f with spent culture supernatant (10^9 to 10^5 CFU cells/ml as indicated) and 500 µl of DMEM were added to each well of the tissue culture plate. The plates were incubated at 37°C in 10% CO_2/90% air for 3 h for *E. coli* and 1 h for *Salmonella*. The monolayers were then washed three times with sterile PBS. Associated bacteria and intestinal cells were dissolved in a 0.2 N NaOH solution. The level of

bacterial association was evaluated by liquid scintillation counting. Each adherence assay was conducted in triplicate with three successive passages of Caco-2 cells by 2 technicians to prevent errors.

- Inhibition of *Salmonella* invasion was performed as follows:

Prior to infection, the Caco-2/TC7 were washed twice with PBS. A exponential culture of *Salmonella* was suspended in DMEM (1.10^8CFU/ml). 500µl of this suspension and 500 µl of *Lactobacillus* (bacteria and spent culture) was added to each well of the tissue culture plate. The plates were incubated for 1h at 37°C in 10% CO_2/90% air and then washed three times with sterile PBS.

Internalization of enteroinvasive bacteria was determined by quantitative determination of bacteria located within the infected monolayers using the aminoglycoside antibiotic assay. After incubation, monolayers were washed twice with sterile PBS and, afterwards, incubated 60 min in a medium containing 100 µg/ml gentamicin. Bacteria that adhere to the Caco-2/TC7 brush border were rapidly killed, whereas those located within Caco-2 cells were not. The monolayer was washed with PBS and lysed with sterilized H_2O. Appropriate dilutions were plated on TSA to determine the number of viable cell-intracellular bacteria by bacterial colony counts.

Results are shown in Figure 3.

I.5 Inhibition assays of bacterial viability, cell-association and cell-invasion of bacteria with Lactobacilli supernatant:

The inhibition of cell-association or invasion of enteroinvasive bacteria by LB-SCS was determined by preincubating the pathogen (10^8 CFU/ml) with control DMEM or concentrated LB-f-SCS for 1 hr at 37°C. After centrifugation (5,500 x g, 10 min. at 4°C), the bacteria were washed with PBS and resuspended in the PBS.

A 18h old of Diffusely Adhesing *E. Coli* (DAEC), or exponential culture of *Salmonella* were centrifuged and suspended in DMEM. Colony count assay were performed by incubating 250µl of pathogens (4.10^8 CFU/ml) with 250µl of *Lactobacillus* supernatant and 500 µl of DMEM 1h at 37°C. Appropriate dilutions were plated on TSA to determine viable bacteria by bacteria colony count.

For inhibition of cell association and cell invasion, cells were infected with 1ml of preincubating (1h, 37°C) pathogens with supernatant. After 1h (*Salmonella*) or 3h (DAEC) incubation at 37°C 10% CO_2, cells were washed three times with PBS. In order to determine the cell-associated bacteria (extracellular + intracellular bacteria), the infected cell monolayers were lysed by adding H_2O. Appropriate dilutions were plated on TSA to determine the number of viable cell-associated bacteria by bacterial colony counts.

Internalization of enteroinvasive bacteria was determined by quantitative determination of bacteria located within the infected monolayers using the aminoglycoside antibiotic assay. After incubation, monolayers were washed twice with sterile PBS and, afterwards, incubated 60 min in a medium containing 100 µg/ml gentamicin. Bacteria that adhere to the Caco-2/TC7 brush border were rapidly killed, whereas those located within Caco-2 cells were not. The monolayer was washed with PBS and lysed with sterilized H_2O. Appropriate dilutions were plated on TSA to determine the number of viable cell-intracellular bacteria by bacterial colony counts.

Each assay was conducted in triplicate with three successive passages of Caco-2 cells.

Results are shown in Figures 2, 4, and 5.

I.6. Antagonistic activity against infected Caco-2 cells:

Activity of LB-f-SCS against intracellular *S. typhimurium* or DAEC was determined using the pre-infected Caco-2. Differentiated Caco-2 cells were infected by *S. typhimurium* SL1344 (1ml, 5.10^7 CFU/ml, 1 hour) or DAEC (1ml, 5.10^7 CFU/ml, 3 hours). For *Salmonella* infection, after two washings of the cells with PBS, the extracellular bacteria were killed by gentamicin (50 µg/ml, 1 hour at 37°C) and the infected cells were washed with PBS to remove the killed bacteria. PBS, MRS (for Man, Rogosa, Sharp) or LB-f-SCS were added apically and the cells were incubated for 1 hour at 37°C. Two hours after the end of treatment, determination of the viable intracellular *S. typhimurium* or viable adherent DAEC was conducted as previously.

Results are shown in Figures 8 and 9.

I.7. Helicobacter pylori:

To test the inhibition of *H. pylori* viability, a 36h old culture in BHI agar was suspended in PBS and centrifuged; bacteria were suspended in BHI broth. Colony count assay were performed by incubating 250µl of pathogens (4.10^8 CFU/ml) with 250µl of *Lactobacillus* supernatant and 500 µl of DMEM 1h at 37°C. Appropriate dilutions were plated on BHI to determine viable bacteria by bacteria colony count.
Results are shown in Figure 6.

Urease activity was determined by a method based on the commercial rapid urease test (RUT ; Jatrox-test ; Röhm-Pharma GmbH, Weiterstadt, Germany) with a sensitivity of 10^2 bacteria. Briefly, 10 µl of *H. pylori* culture were added to 1ml of the reaction solution (urea 0.1g/ml [wt/v] containing 17 µg of phenol red/ml [wt/v] as a pH indicator). The development of urease activity was measured as a function of time by a spectrophotometric analysis at 550 nm.

Results are shown in Figure 7.

II. RESULTS

A. Bacteria and supernatants :

II.A. 1. Bacterial adhesion :

Figure 1 shows that the living LB-f strain display a dose-dependent adhesiveness onto cultured human intestinal cells. In addition, killed LB-f bacteria display the same capacity of adhesion, although a slight decrease in level of adhesion was observed as compared with living bacteria.

II. A.2. Inhibitory activity against adhesion of radiolabeled enterovirulent bacteria:

Figure 2 shows that both culture of living and killed LB-f bacteria exerted an inhibitory activity against adhesion of diffusely adhering *E. coli* (DAEC) strain C1845 onto cultured human intestinal cells. This activity is dose-dependent. Moreover, the same level of inhibitory activity is observed for control in DMEM or MRS-HCL pH 4.5.

II.A.3. Inhibitory activity against invasion of *Salmonella enterica* serovar Typhimurium:

Figure 3 shows that culture of living LB-f bacteria inhibits the cell-invasion by *Salmonella* serovar Typhimurium within cultured human intestinal cells. Killed LB-f bacteria exerted an inhibitory activity.

B. Supernatants :

II.B.1. Antibacterial activity :

a) against *Salmonella enterica* serovar Typhimurium.

Figure 4 shows that the spent culture supernatant (SCS) of living LB-f bacteria and the heated SCS decreased the viability of *S. typhimurium* strain. This activity is similar that activity of LB strain. The SCS of living LB-f bacteria and the heated SCS decreased the adhesion onto and internalization within cultured human intestinal cells by *S. typhimurium* strain. These activities are similar that activities of LB strain.

b) against DAEC :

Figure 5 shows that the SCS of living LB-f bacteria and the heated SCS slightly decreased the viability of diffusely adhering *E. coli* (DAEC) strain C1845 and strongly decreased adhesion of pathogenic *E. coli* onto cultured human intestinal cells.

c) against *Helicobacter pylori* :

Figure 6 shows that the SCS of living LB-f bacteria and the heated SCS strongly decreased the viability of *Helicobater pylori*.

Figure 7 shows that the SCS of living LB-f bacteria and the heated SCS strongly decreased the urease activity of *Helicobater pylori*.

II.B.2. Cell-association and cell-invasion of enteroinvasive bacteria:

a) Cell-invasion of *Salmonella enterica* serovar Typhimurium:

Figure 8 shows that the SCS of living LB-f bacteria and the heated SCS strongly decreased the level of living, internalized *S. typhimurium* bacteria within primarily infected cultured human intestinal cells.

b) Cell-association of DAEC C1845 :

Figure 9 shows that the SCS of living LB-f bacteria and the heated SCS strongly decreased the level of adhering, living diffusely adhering *E. coli* C1845 bacteria into primarily infected cultured human intestinal cells.

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CLAIMS

1. A *Lactobacillus fermentum* strain (LB-f strain), deposited at the CNCM (Paris, France) on March 27, 2003, under registration number I-2998.

2. The LB-f strain according to claim 1, having at least the following phenotypic characters:

- regular, non sporing, Gram-positive rod;
- heterofermenting;
- catalase negative;
- L (+)-lactic acid-producing.

3. The LB-f strain according to claim 1 or 2, having a 16S rDNA sequence comprising a nucleotide sequence selected from:

- SEQ ID No. 1 ;
- its complementary sequence ; and
- sequences identical at least at 98.1% to SEQ ID No. 1 or to its complementary sequence.

4. The LB-f strain according to claim 3, wherein said sequences are identical at least at 98.5%, and preferably at least at 99% to SEQ ID No. 1 or to its complementary sequence.

5. The LB-f strain according to claim 4, wherein said sequences are identical at least at 99.5%, and preferably at least at 99.8% to SEQ ID No. 1 or to its complementary sequence.

6. A method for cultivating a *Lactobacillus fermentum* strain (LB-f strain) according any of claims 1 to 5, comprising at least:

a) providing a culture medium containing at least lactose and yeast extract;

b) cultivating said LB-f strain in said culture medium under fermenting conditions; and

c) recovering the thus obtained culture of the LB-f strain.

7. The method according to claim 6, wherein said culture medium contains lactose at a concentration range of about 50 to about 100 g/l.

8. The method according to claim 6 or 7, wherein said culture medium contains yeast extract at a concentration range of about 5 to about 20 g/l.

9. The method according to any of claims 6 to 8, wherein said fermenting conditions in step b) are pH-regulated, said pH ranging between about 4.5 and 5.5.

10. The method according to any of claims 6 to 9, further comprising separating the biomass from the culture supernatant (LB-f-SCS) by centrifugating said culture of LB-f strain recovered in step c).

11. The method according to claim 10, further comprising recovering said biomass and/or said LB-f-SCS.

12. A *Lactobacillus fermentum* culture supernatant (LB-f-SCS) obtainable by a method according to claim 11.

13. A *Lactobacillus fermentum* strain (LB-f strain) according to any of claims 1 to 5, for use as a medicine.

14. The LB-f strain according to claim 13, wherein said medicine is used for preventing and/or treating gastrointestinal disorders.

15. The LB-f strain according to claim 14, wherein said gastrointestinal disorders are selected from ulcers and infections due to *Helicobacter pylori*, intestinal inflammatory diseases, such as ulcerous colitis, Crohn's disease and pouchitis, irritable bowel syndrome, steatohepatitis, hepatic steatosis, and infectious diarrhoea.

16. A *Lactobacillus fermentum* culture supernatant (LB-f-SCS) according to claim 12, for use as a medicine.

17. The LB-f-SCS according to claim 16, wherein said medicine is used for preventing and/or treating gastrointestinal disorders.

18. The LB-f-SCS according to claim 17, wherein said gastrointestinal disorders are selected from ulcers and infections due to *Helicobacter pylori*, intestinal inflammatory diseases, such as ulcerous colitis, Crohn's disease and pouchitis, irritable bowel syndrome, steatohepatitis, hepatic steatosis, and infectious diarrhoea.

19. Use of a *Lactobacillus fermentum* strain (LB-f strain) according to any of claims 1 to 5, for the manufacture of a medicine for preventing and/or treating gastrointestinal disorders.

20. Use of a *Lactobacillus fermentum* culture supernatant (LB-f-SCS) according to claim 12, for the manufacture of a medicine for preventing and/or treating gastrointestinal disorders.

21. The use according to claim 19 or 20, wherein said gastrointestinal disorders are selected from ulcers and infections due to *Helicobacter pylori*, intestinal inflammatory diseases, such as ulcerous colitis, Crohn's

disease and pouchitis, irritable bowel syndrome, steatohepatitis, hepatic steatosis, and infectious diarrhoea.

22. Use of a *Lactobacillus fermentum* strain (LB-f strain) according to any of claims 1 to 5, as a dietary product.

23. Use of a *Lactobacillus fermentum* culture supernatant (LB-f-SCS) according to claim 12, as a dietary product.

24. A pharmaceutical composition comprising a *Lactobacillus fermentum* strain (LB-f strain) according to any of claims 1 to 5, and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition according to claim 24, wherein said LB-f strain is present in an amount from about 10^9 to about 10^{12} bacteria/g, preferably from about 10^9 to about 10^{11} bacteria/g, and more preferably from about 10^9 to about 10^{10} bacteria/g.

26. A pharmaceutical composition comprising a *Lactobacillus fermentum* culture supernatant (LB-f-SCS) according to claim 12, and a pharmaceutically acceptable carrier.

27. The pharmaceutical composition according to claim 26, wherein said LB-f-SCS is present in an amount of at least about 100 mg per gram of composition.

28. The pharmaceutical composition according to any of claims 24 to 27, wherein said composition is ingestible.

29. The pharmaceutical composition according to claim 28, wherein said composition is in a form selected from tablets, liquid bacterial

suspensions, dried oral supplements, wet oral supplements, dry tube feeding, wet tube feeding.

30. A dietary composition comprising a *Lactobacillus fermentum* strain (LB-f strain) according to any of claims 1 to 5, and a food carrier.

31. The dietary composition according to claim 30, wherein said LB-f strain is present in an amount from about 10^5 to about 10^9 bacteria/g, preferably from about 10^6 to about 10^8 bacteria/g, and more preferably from about 10^6 to about 10^7 bacteria/g.

32. A dietary composition comprising a *Lactobacillus fermentum* culture supernatant (LB-f-SCS) according to claim 12, and a food carrier.

33. The dietary composition according to claim 32, wherein said LB-f-SCS is present in an amount of less than about 100 mg per gram of composition.

34. The dietary composition according to any of claims 30 to 33, wherein said dietary composition is ingestible.

35. The dietary composition according to claim 34, wherein said composition is selected from milk, yogurt, curd, cheese, fermented milks, fermented milk-based products, ice-creams, fermented cereal-based product, milk-based powders, infant formulae.

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36. A method for treating or preventing gastrointestinal disorders in a mammal, especially a human, in need of such treatment, said method comprising:

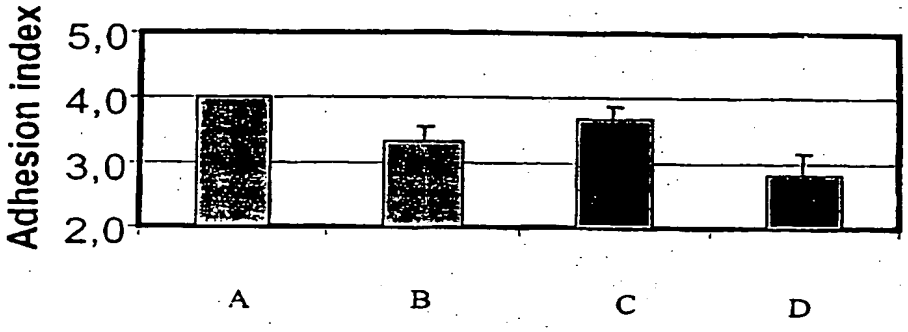
administering to said mammal a pharmaceutically effective amount of a medicine selected from the group of:

- a *Lactobacillus fermentum* strain (LB-f strain) according to any of claims 1 to 5; or
- a *Lactobacillus fermentum* culture supernatant (LB-f-SCS) according to claim 12; or
- a pharmaceutical composition according to any of claims 24 to 29.

37. The method according to claim 36, wherein said gastrointestinal disorders are selected from ulcers and infections due to *Helicobacter pylori*, intestinal inflammatory diseases, such as ulcerous colitis, Crohn's disease and pouchitis, irritable bowel syndrome, steatohepatitis, hepatic steatosis, and infectious diarrhoea.

38. The method according to claim 36 or 37, wherein said medicine is administered orally.

39. The method according to claim 38, wherein said medicine in a form selected from tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding, wet tube feeding.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B
C
D

Fig. 1

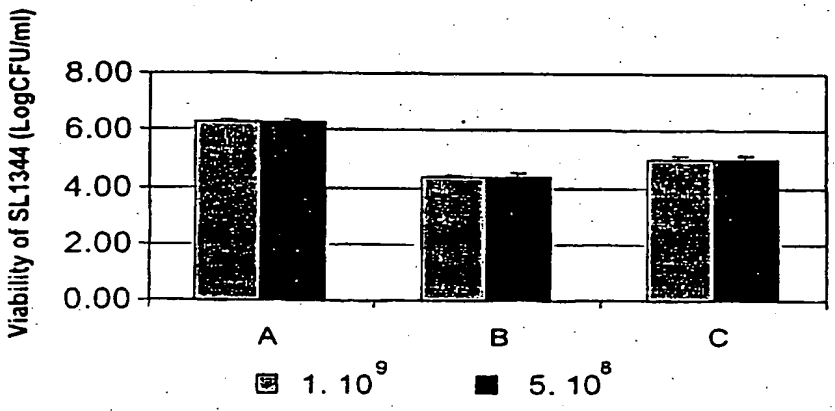
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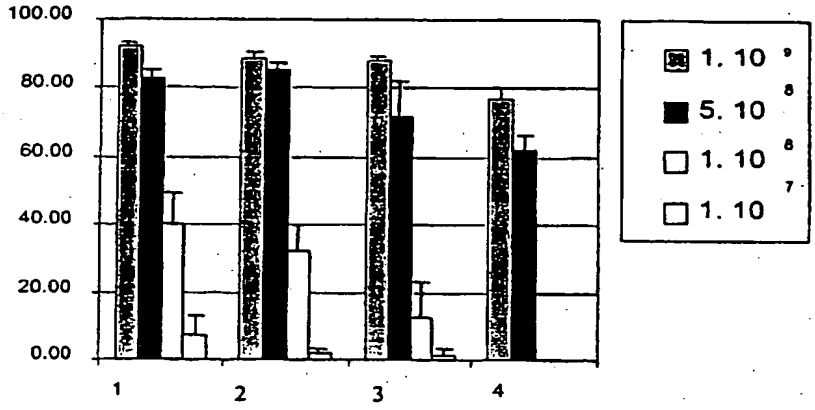
Fig. 3

A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B

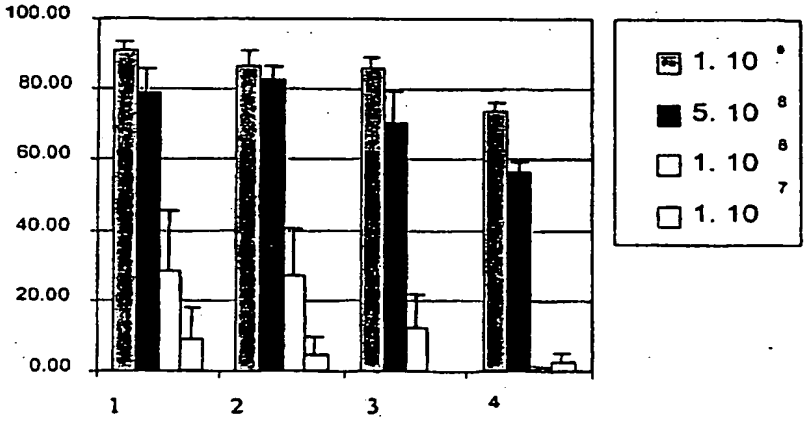
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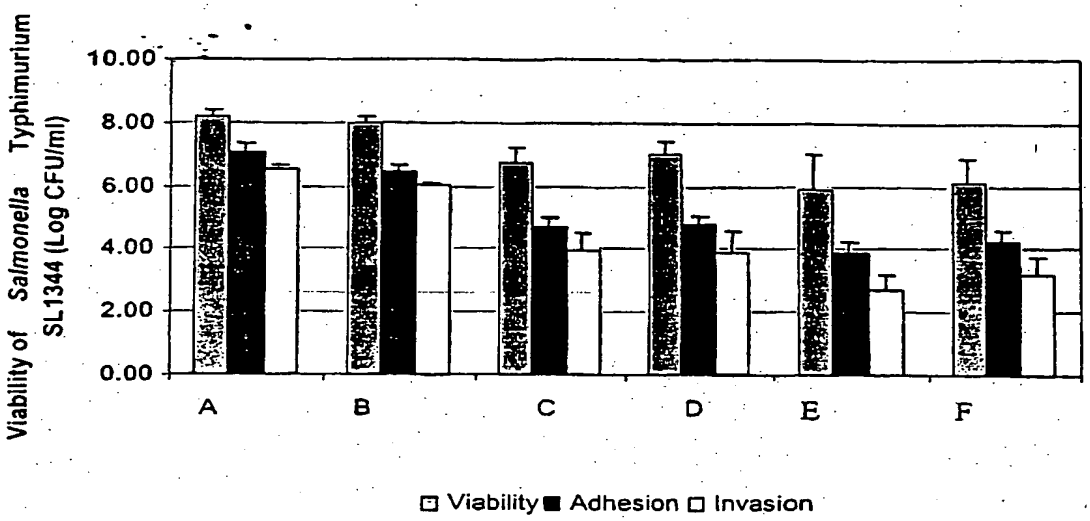
Fig. 2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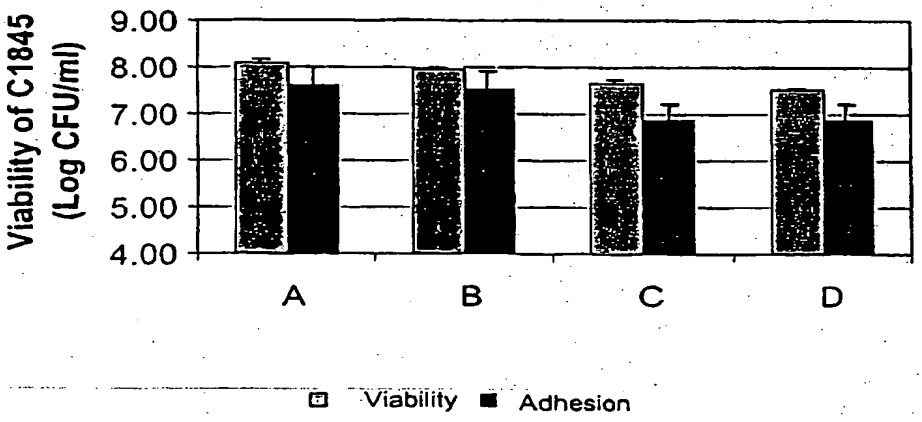
D
E
F
Viability Adhesion Invasion

Fig.4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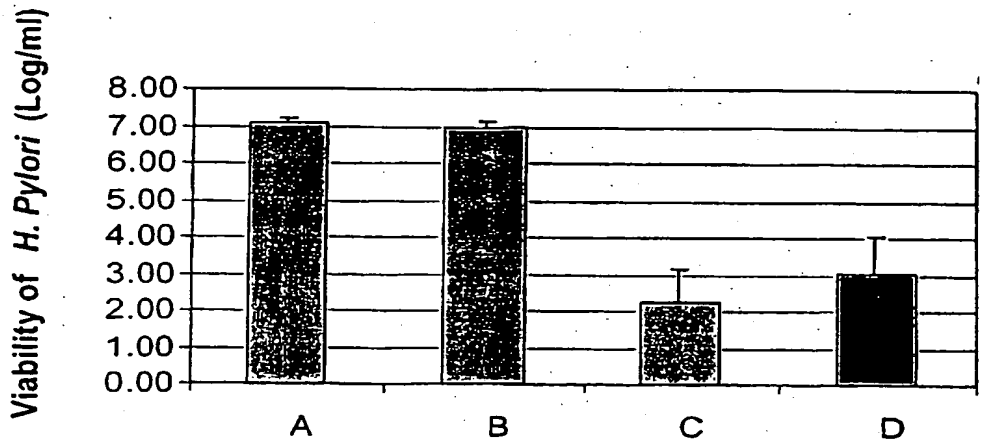
C
D
Viability Adhesion

Fig. 5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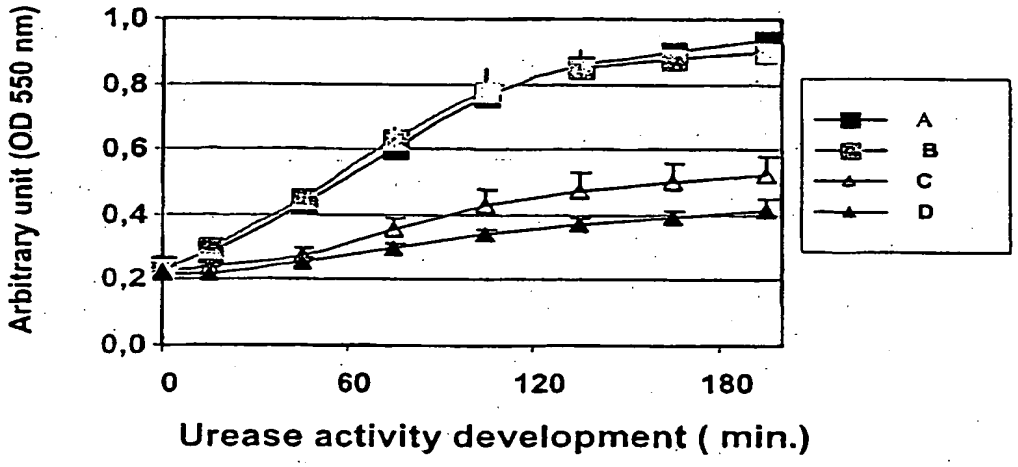
C
D

Fig. 6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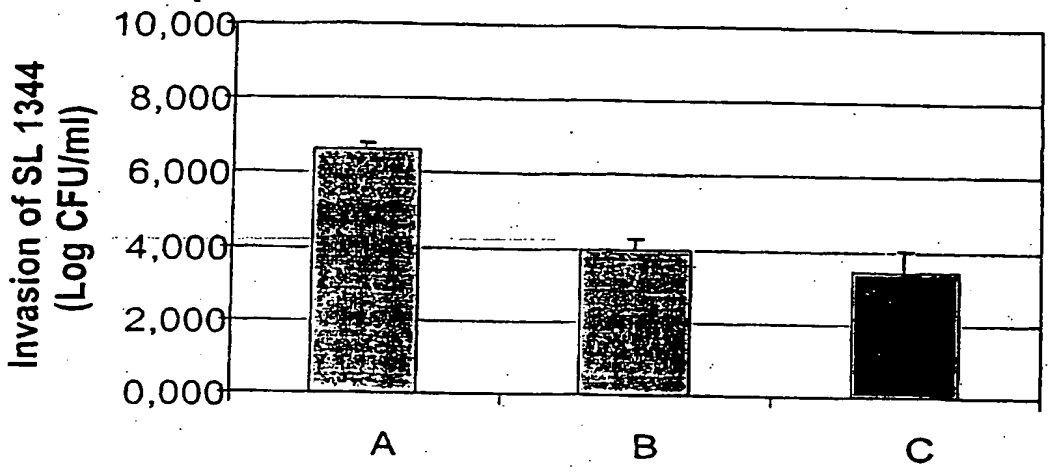
C
D

Fig. 7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A
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C

Fig. 8

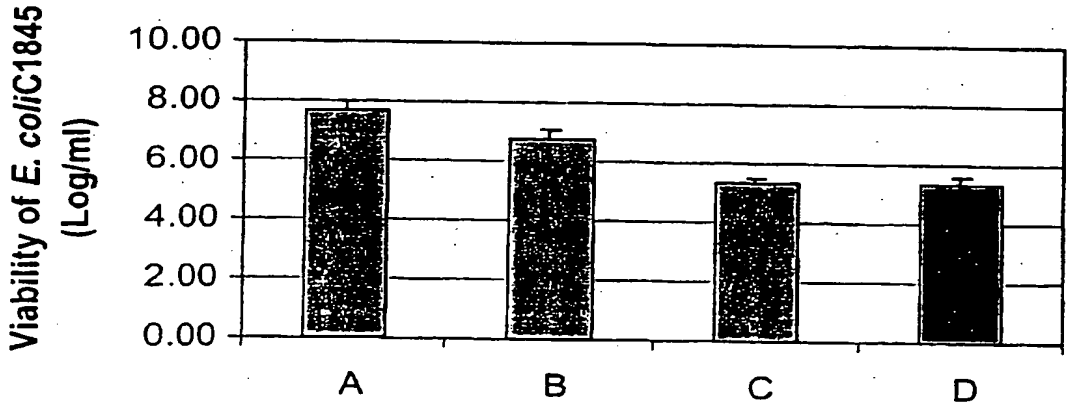
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Fig. 9

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INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 A61K35/74 A23C9/152 A23L1/30 A23C9/123 C12R1/225

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K A23L A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, MEDLINE, SEQUENCE SEARCH, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02 45727 A (LEE YEONHEE ;PAEK KYUNGSOO (KR); PLBIO CO LTD (KR)) 13 June 2002 (2002-06-13) cited in the application	1-5, 13-15, 19,21, 22,24, 25, 28-31, 34-39
Y	page 3, line 12 -page 5, line 14	6-12, 16-18, 20,23, 26,27, 32,33
	page 7, line 8 -page 14, line 2; example 1 * see Sequence Listing: Seq ID NOs: 5 and 6	
	-/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 2003	08/10/2003

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer
Donath, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03 002131 A (ANNUK HEIDI ;SONGISEPP EPP (EE); ZILMER MIHKEL (EE); KULLISAAR TII) 9 January 2003 (2003-01-09) page 3, line 33 -page 7, line 14 page 11, line 1 -page 13, line 13	1-3, 13-15, 19,21, 22,24
X	RANDAZZO CINZIA L ET AL: "Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 68, no. 4, April 2002 (2002-04), pages 1882-1892, XP009017967 April, 2002 ISSN: 0099-2240 page 1883 -page 1884 'Materials and Methods' tables 2,4 -& DATABASE EMBL 'Online! "Uncultured bacterium clone 60CR 16S ribosomal RNA gene, complete sequence" Database accession no. AF349926 XP002255556 the whole document	1-5
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